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Prospective Administration of Anti-NGF Treatment Effectively Suppresses Functional Connectivity Alterations Following Cancer-Induced Bone Pain in Mice

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Abstract

Cancer-induced bone pain is abundant among advanced stage cancer patients and arises from a primary tumor in the bone or skeletal metastasis of common cancer types such as breast, lung or prostate cancer. Recently, antibodies targeting nerve growth factor (NGF) have been shown to effectively relieve neuropathic and inflammatory pain states in mice and in humans. While efficacy has been shown in mice on a behavioral level, effectiveness in preventing pain-induced functional rearrangements in the central nervous system has not been shown. Therefore we assessed longitudinal whole-brain functional connectivity using resting-state fMRI in a mouse model of cancer-induced bone pain. We found functional connectivity between major hubs of ascending and descending pain pathways such as the periaqueductal gray, amygdala, thalamus as well as cortical somatosensory regions to be affected by a developing cancer pain state. These changes could be successfully prevented through prospective administration of a monoclonal anti-NGF antibody (mAb911). This indicates efficacy of anti-NGF treatment to prevent pain-induced adaptations in brain functional networks following persistent nociceptive input from cancer-induced bone pain. Additionally, it highlights the suitability of resting-state fMRI readouts as an indicator of treatment response on the basis of longitudinal functional network changes.

Keywords: resting-state fMRI; Chronic Pain; metastatic bone cancer; Nerve growth factor; pharmacological modulation

1. Introduction

Common cancer types like breast, lung and prostate cancer have a high predisposition of forming skeletal metastasis in bones like femur, tibia, ribs and vertebrae [11]. Tumor formation in bones frequently leads to anemia, increased risk of fracture, susceptibility to infections and in many cases to a severe pain state, impairing patient's quality of life. Cancer-induced bone pain is a complex pain state known to cause persistent pain, movement evoked pain and severe episodes of breakthrough pain, which are particularly difficult to manage [30]. Commonly, ongoing cancer pain is managed adhering to the World Health Organization's analgesic ladder in combination with adjuvant therapies such as bisphosphonates, non-steroidal anti-inflammatory drugs, opioids, radiotherapy or surgical interventions [51]. While these therapies often exert severe side-effects especially upon long-term use, they also fail to address spontaneous breakthrough pain. Therefore effective treatment of cancer-induced bone pain is still not available.

In order to better understand mechanisms involved, a preclinical model of bone cancer pain has been developed by orthotopic implantation of tumor cells [12,24]. The model was shown to reproduce clinical symptoms of cancer-induced bone pain including spontaneous and evoked pain, correlating with tumor growth and cancer-induced bone remodeling [30]. Besides osteoclast mediated bone resorption, tumor acidosis and mediators released by tumor-associated stromal cells, tumor-induced damage to peripheral nociceptors was found to be a major contributor of cancer pain [40,46]. Nociceptive Aδ- and C-fibers innervating the adult bone are to the largest extent tyrosine kinase A (TrkA) positive, target receptor of the nerve growth factor (NGF) [31]. NGF sequestering antibodies for treating cancer pain were found to be highly effective in reducing tumor-induced nerve sprouting, neuroma formation and bone cancer pain in mice [29,47]. Effective relieve from skeletal pain was further indicated in clinical studies in different disease states like osteoarthritis and chronic lower back pain [9].

Along with these peripheral effects, cancer-induced bone pain show very distinct neurochemical alterations in the central nervous system (CNS) at the level of the spinal cord such as overexpression of dynorphin and c-FOS, as well as actrocyte hypertrophy [25]. These effects could partly be prevented through application of anti-NGF treatment [47]. While anti-NGF treatment efficacy is largely described assessing peripheral, behavioral or spinal cord level readouts, its effects on the brain remain unknown. As the conscious experience of pain is ultimately generated in the brain, assessing peripheral effects of anti-NGF treatment on the brain in a cancer pain state might give further insights into how effective pain relieve is.

Therefore, we assessed whole brain functional connectivity (FC) using resting-state functional magnetic resonance imaging (rs-fMRI) in a mouse model of bone cancer pain. Rs-fMRI infers FC based on the synchronicity of spontaneous activity across brain regions [4,5,13,32] and poses a unique window for assessing the functional state of the brain and how it is affected upon a disease state [16,18,50]. By assessing longitudinal FC, we evaluated efficacy of the anti-NGF antibody mAb911 to modulate changes in brain networks following a cancer-induced bone pain state in mice.

2. Material and Methods

Animals & Experimental Design

Animal experiments were conducted in accordance with regulations of the Cantonal Veterinary Office in Zürich, Switzerland. Female C57BL/6 mice at 8-10 weeks of age were obtained from Janvier Labs (Laval, France). Mice were held in individually ventilated cages in a 12/12h alternating light/dark cycle at 22-24°C with unrestricted access to food and water, experiments were conducted during light cycle. They were allocated to groups, and groups were allocated to

cages in a randomized fashion using R's sample function [42]. Separation of groups by cages was decided to prevent social transfer of pain which has been previously described [48]. Female mice were chosen due to the higher prevalence of females to develop a chronic pain state [22].

fMRI experiments were carried out at baseline (BL) before tumor inoculation (0d.p.i.), 10 and 20 days post injection (d.p.i.) of the tumor cells. Anti-NGF treatment and behavioral readouts of pain were carried out in parallel. Treatment and behavioral readouts were initiated 1 week post tumor inoculation (8d.p.i.). Thereby, anti-NGF treatment was injected after completion of behavioral experiments. Experimental groups used in this study and their characteristics are indicated in Table 1. An outline of the experimental design is indicated in Figure 1.

Mouse Model of Bone Metastasis

The mouse model of chronic pain from bone metastasis was adopted form previous published procedures and refined for our purposes [23,26]. In short, EO771 breast cancer cells were cultured to 80% confluency in RPMI medium supplemented with 10% fetal bovine serum at 37°C. Mice of the tumor-bearing groups (Tumor+Vehicle and Tumor+anti-NGF) were injected with 10^5 cells in 10µl in the medulla of the right tibia while mice of the control-groups (Sham+Vehicle and Sham+anti-NGF) were injected with 10µl PBS instead. Therefore, mice were anesthetized at 3% isofluorane in a 1:5 mixture of O2/air, the hair was removed and skin disinfected on the injected leg. To access the medullary space, a hole was drilled through the tibial plateau using a 27-G needle. Proper location of the needle was verified in two planar x-ray radiographs (35kW/10s, 4x geometric magnification) of a 90° angle using a Faxitron MX-20 digital radiography system. Cells where then slowly injected using a 27-G Kel-F hub needle on a Hamilton syring and 1min time was given for cells to accommodate in the medullary space before removing the needle. Post-operative pain was managed by subcutaneous (s.c.) injection of Temgesic (60µg/kg). During the whole procedure, mice were placed on a feedback-controlled heating mat to maintain physiological body temperature.

Behavioral Readouts of Spontaneous Pain Behavior

Behavioral experiments were conducted in a blinded fashion every 5^{th} day, starting 1 week after tumor inoculation (8, 13 and 18d.p.i.). Thereby, the animals were placed on a home-built mesh stand, enclosed in a 10x10x20cm compartment. Blinding was ensured by random placing of the animals in the absence of the experimenter conducting behavioral studies. All mice were habituated for 2x1h before start of the study. Before every experiment, mice were additionally habituated for 30min. Guarding and flinching behavior was analyzed over the period of 2min by video recording. Guarding was defined as holding the paw aloft while ambulatory, flinching was defined as holding the paw aloft while steady according to previously published procedures [6,29]. Number of guarding and flinching incidences was assessed from the video after the experiment.

Animal Preparation and Anesthesia

For induction of anesthesia, mice were anesthetized using 4% isofluorane in a 1:5 mixture of O2/air for 4min before endotracheal intubation using PE-50/10 polyethylene tubing while maintaining anesthesia at 2%. Mice where cannulated intravenously (i.v.) in the lateral tail vein using a the tip of a 30-G needle, the animal was placed on the MRI-support before application of pancuronium bromide (0.05mg/kg, s.c.) and medetomidine hydrochloride (0.1mg/kg, i.v.) bolus. After 5min, isoluorane anesthesia was reduced to 0.5% and combined with a medetomidine hydrochloride infusion (0.05mg/kg/h, i.v.) during the course of acquisition. During experiments, mice were mechanically ventilated using a small animal ventilator at a rate of 80breaths/min with a cycle of 25% inhalation and 75% exhalation and 1.8ml inspiration volume. Functional data acquisition was consistently started 15min after the start of medetomidine infusion in order to ensure reproducible physiological states. In order to reduce motion artefacts, mouse heads were fixed using bite and ear bars. Body temperature was monitored using a rectal probe and maintained at 36.5±0.1°C during the course of the experiments. Upon termination of MRI experiments, animals were recovered on a heating map while still ventilated at 1% isofluorane until the effect of pancuronium bromide has worn off.

Anti-NGF Treatment using Murine anti-NGF Monoclonal Antibody mAb911

Murine monoclonal anti nerve growth factor (NGF) antibody mAb911 was kindly provided by Pfizer Inc. (Groton, CT, USA). Treatment regime and dosing were adopted from previous publications, demonstrating the effectiveness of mAb911 to reduce cancer-induced bone pain [47]. Anti-NGF treatment (10mg/kg, i.p.) was initiated prospectively at 8d.p.i. and repeated in intervals of 5days following behavioral experiments.

MRI Acquisition Protocol

Magnetic resonance imaging (MRI) experiments were conducted using a Biospec 94/30 small animal MR system with a 30cm horizontal bore operating at 400MHz (9.4T). For excitation and signal reception, a cryogenic quadrature transmit/receive surface coil was used.

Resting-state fMRI: Functional data was acquired using a gradient-echo echo planar imaging (GE-EPI) sequence sensitive to the blood-oxygen level dependent (BOLD) contrast with the following parameters: Field of view (FOV) = 16x8mm2 and Matrix size (MTX) = 80x40, resulting in 200x200µm2 in-plane resolution, 18 slices, slice thickness (SLTH) = 0.5mm, repetition time (TR) = 1000ms, echo time (TE) = 12ms, bandwidth (BW) = 200kHz and number of averages (NA) = 1. Acquisition time was 15min20sec (920 repetitions) of which the first 20 were discarded and 900 were used for subsequent analysis. To prevent fold-over artefacts, one saturation slice was positioned at the base of the brain.

Data Processing and Statistical Analysis

Functional scans were normalized to a MRI template (Australian Mouse Brain Mapping Consortium, http://www.imaging.org.au/AMBMC) using linear affine and non-linear greedy SyN transformation (ANTs V2.1, http://picsl.upenn.edu/software/ants/). Independent functional components (ICs) were extracted on an individual level using MELODIC (Multivariate Exploratory Linear Optimized Decomposition of Independent Components [3]), taking the mean EPI image as a reference. This process included 0.01Hz high-pass filtering of the 4D dataset, spatial smoothing with a 2x2mm^2 kernel and head motion correction using MCFLIRT [27]. Independent components were classified into physiological and non-physiological components using an in-house classifier [52], non-physiological components in the BOLD signal were regressed out using FIX (FMRIB's ICA-based Xnoiseifier [20,44], v1.06). Classifier accuracy was tested using a subset of 10 baseline scans, yielding an accuracy A (A=TNR+TPR/2) of 72.5%, TNR and TPR indicating true positive and true negative discovery rate, respectively, for the applied threshold during analysis. Group-level independent components of baseline scans were used as naïve animal reference resting state networks (RSN) to analyzed projection regions of seeds in the seed-based analysis approach.

Region of interest (ROI) -based network analysis of whole brain connectivity was performed using a ROI-template comprising 75 anatomical ROIs based on the reference atlas of the Allen Institute for Brain Sciences (AIBS). Using fslmaths, average BOLD-timeseries were extracted from the normalized, filtered data. Pairwise Pearson's correlation values were calculated between all ROI combinations to obtain subject and session specific correlation matrices. Fisher's Z transformed correlation matrices were used in a linear mixed model analysis to test for group and session interaction effects (R, lme4 package). For the seed-based analysis, average timeseries were extracted from seeds and used in a general linear model (GLM) analysis (fsl_glm) as a regressor. Resulting subject and session level voxel-wise Z-score maps of seed projections were subsequently analyzed within target ROIs using previously described resting state networks as ROIs in a linear mixed model analysis, accounting for multiple comparisons (R, lme4, multcomp packages).

Behavioral readouts of pain were analyzed using multiple t-tests (one per time-point), statistical significance was corrected for multiple comparisons using the Holm-Sidak method. Descriptive statistics are indicated as means ±1 standard deviation (SD) in all plots with a statistical significance level of $P<0.05$.

3. Results

Behavioral Readouts of Spontaneous Pain

A linear mixed model analysis indicated significant group and session interactions in the Tumor-Vehicle vs Control-Vehicle ($P<0.0003$ for guarding, $P<0.0001$ for flinching) and Tumor-anti-NGF vs Control-Vehicle ($P<0.04$ for guarding and $P<0.1$ for flinching). Control-anti-NGF vs Control-Vehicle was not significant in both readouts. Post-hoc, we performed multiple t-tests, one per session, to determine statistically significant differences at each time point (Figure 2). Guarding behavior was found to be elevated in Tumor+Vehicle animals at 13 and 18d.p.i., while only a small increase in guarding was observed at 18d.p.i. in Tumor+anti-NGF mice. There was a significant treatment-effect visible between these two groups at 13 and 18d.p.i.. Sham operated animals did not show any significant guarding behavior irrespective of whether they were treated with vehicle or anti-NGF. Similar results have been obtained regarding flinching behavior, which was found to be elevated in Tumor+Vehicle animals at 18d.p.i. compared to any other group. In particular, Tumor+anti-NGF animals did not develop any significant flinching behavior resulting in a significant treatment-effect between for tumor mice at 18d.p.i.. Behavioral readouts of spontaneous pain behavior indicate development of pain in Tumor+Vehicle animals. This behavior could be largely prevented through prospective treatment using anti-NGF antibody mAb911 with only little residual guarding behavior displayed at 18d.p.i. in Tumor+anti-NGF animals.

ROI-based Network analysis

ROI-based analysis of longitudinal rs-fMRI data revealed distinct rearrangement of functional connectivity (FC) patterns in tumor-bearing as compared to sham-operated mice upon vehicle treatment (Figure 3, upper triangle). Interactions of amygdallar nuclei (Amg) with thalamic (Th) as well as midbrain (Mb) regions were found to be most significantly affected. A closer look on midbrain structures revealed FC changes to be mostly confined in motor-related areas and the periaqueductal gray. In addition, FCs to cortical areas (Ctx) were found to be affected; in particular interactions with temporal associative (TAc) / insular (IC) regions and the cingulate cortex (Cg). Furthermore, FC between the thalamus and cortical areas were specifically affected in somatosensory as well as temporal associative / insular regions (Supplementary Fig 1, available at http://links.lww.com/PAIN/A659). Less distinct but still significant changes were additionally observed between striatal (Str) and midbrain as well as thalamic regions.

Prospective treatment of tumor-bearing mice using mAb911 successfully prevented previously observed effects of persistent pain on FC (Figure 3, lower triangle). Few remaining significant alterations were found in deep nuclei comprised in mid- and hindbrain (Hb) regions. The distinct patterns of altered connectivities associated with tumor development were virtually absent in Tumor+anti-NGF animals.

Significant treatment effects of mAb911 are indicated in Supplementary Figure 2 (upper triangle), comparing animals of the Tumor+Vehicle and Tumor+anti-NGF groups (available at http://links.lww.com/PAIN/A659). FCs responding to treatment overlapped to a large extent with FCs exhibiting significant changes due to tumor development (Fig. 3; upper triangle). In order to exclude intrinsic effects of mAb911 treatment we compared FC in Sham+Vehicle and Sham+anti-NGF mice (Supplementary Figure 2, lower triangle, available at http://links.lww.com/PAIN/A659). We did not find significant differences between the groups except minor effects on FC between midbrain and striatal and amygdallar regions indicating low significance and random distribution across the brain.

In summary, we found distinct patterns on FC alterations within main regions of ascending and descending pain pathways in the untreated tumor-bearing group. These FC rearrangements could be largely prevented following prospective mAb911 treatment and significant treatment effects were found. Intrinsic effects of mAb911 treatment on brain functional networks appear to be largely absent, a least for the treatment regime applied.

Seed-based analysis

For analyzing effects of cancer-induced bone pain on FC and their modulation by anti-NGF treatment, a seed-based analysis was carried out using the main nuclei of ascending and descending nociceptive pathways as seed regions. The regions were chosen according to previously described results of ROI-based network analysis (Figure 3) in both hemispheres separately, in nuclei of the amygdala, anterior and posterior part of the periaqueductal gray, thalamus and the motor-related portion of the superior colliculus. Alterations of regional seed FC was analyzed within bilateral target reference resting state networks derived from naïve animals using independent component analysis [52]. Statistical significance was assessed using a linear mixed model analysis testing for significant group and session interaction, corrected for multiple comparisons was conducted using false discovery rate (FDR) correction.

Significant effects were found for FC between the amygdallar seed (lAmg) contralateral to the tumor site and thalamic (Th, p=0.004), dorsal hippocampal (dHp, p=0.006) and somatosensory (B1c, p=0.021) areas (Figure 4). Furthermore, FC between the anterior periaqueductal gray seed (aPAG) and barrelfield 2 cortex (B2c, p=0.032), posterior periaqueductal gray seed (pPAG) and limb cortex (Lc, p=0.032) as well as ipsilateral motor related superior collicular seed (rSCm) seed and the amygdala (Amg, p=0.035) were found to be significantly affected in the Tumor+Vehicle group. These changes in FC could be prevented by treatment with anti-NGF antibodies as the Tumor+anti-NGF group did not display altered FCs in these regions when compared to Sham+Vehicle animals. FC of the contralateral amygdallar seed to the cingulate cortex indicated the same trend, though the effect did not reach statistical significance (p=0.136, data not shown). As a control, FC was analyzed using a seed in the ipsilateral amygdala (rAmg), a region that does not receive direct nociceptive input. FC from the right amygdala to the thalamus, barrelfield 1

cortex and dorsal hippocampus, the corresponding regions to those displaying altered FC to the left amygdala, was not affected by the developing tumor in the right leg (Th p=0.334, B1c p=0.479 and dHp p=0.307, Supplementary Figure 3, available at http://links.lww.com/PAIN/A659) as Z-scores in respective regions were found to be stable in all groups and measurement sessions. Assessment of treatment effects at 20d.p.i revealed that mAb911 administration prevented FC changes between the left amygdallar seed and the dorsal hippocampus (p=0.031) and barrelfield 1 cortex (p=0.013). Furthermore, FC between the anterior periaqueductal gray seed and the barrelfield 2 cortex (p=0.004) as well as between the posterior periaqueductal gray seed and the limb cortex (p=0.012) were significantly modulated by the treatment. Significant FC changes upon a persistent pain from bone cancer which could be prevented through mAb911 administration are schematically visualized in Figure 5. Affected connectivities are depicted with respective differences in Z-scores (ΔZ) comparing Tumor+Vehicle vs Sham+Vehicle animals, indicating effect-sizes of FC differences induced by bone cancer pain which could be prevented through mAb911 administration.

For visualizing treatment effects of mAb911, FC at 20d.p.i. was compared in animals of the Tumor+Vehicle and Tumor+anti-NGF groups versus the Sham+Vehicle group using an unpaired t-tests on a voxel by voxel basis (fsl-randomise). Uncorrected t-statistical maps visualized previously described effects indicate voxels within a reference resting state network, where seed FC is increasing (>) compared to Sham+Vehicle controls (Figure 6). While Tumor+Vehicle animals consistently indicate reduced FC between seeds and reference resting state networks compared to Sham+Vehicle animals, this reduction in FC could be significantly prevented in the Tumor+anti-NGF group.

4. Discussion

Using longitudinal rs-fMRI and behavioral readouts of spontaneous pain in a mouse model of cancer pain from bone metastasis we assessed efficacy of the NGF sequestering antibody mAb911 to prevent cancer-induced bone pain. The primary molecular target of NGF is TrkA, to which it binds with a high affinity, leading to the formation of homodimers [10]. More recent findings suggest NGF binding to $p75^{NTR}$, forming a heterodimer which is presented to TrkA [1]. Formation of such homo- and heterodimers leads to activation of secondary-messenger cascades, modulating expression patterns of receptors and ion channels on peripheral nerve endings and induce sensitization through a variety of neurotransmitters [35]. Additionally, NGF induces sprouting and hyper-innervation of bones in metastatic bone diseases leading to the development of skeletal pain [33]. While there is some knowledge on pathways affected by NGF, the exact molecular mechanisms and cell-type specific expression patterns are still not clear.

The effects of NGF on peripheral nociceptors indicates fast sensitization of neurons towards chemical, mechanical and thermal stimuli, which translate into prolonged alterations fibers affecting CNS function [2]. Anti-NGF treatment has been shown to prevent such effects, as typical neurochemical changes in the spinal cord as a result of cancer induced bone pain could be prevented [47]. Furthermore, anti-NGF treatment has been shown to be effective in a rat model of spinal cord injury, in which anti-NGF antibodies successfully suppressed mechanical hyperalgesia and increased responsiveness of wide dynamic range neurons in the spinal cord [21]. Nevertheless, studies indicating direct effects of NGF and anti-NGF treatment on the CNS remain sparse, probably due to the fact that target receptor expression is largely limited to peripheral nociceptors both in mammals and rodents [36,49]. Moreover, direct effects are expected to be minimal due to poor CNS penetration of IgG molecules (>0.1% [39]), whereas circulating concentrations are much higher. Therefore, effects of anti-NGF treatment in the CNS arise most likely from altered peripheral nociceptive processing rather than direct central action.

Along the lines of these results, our findings indicate efficacy of anti-NGF treatment to prevent FC in the brain following cancer-induced bone pain. We have shown alterations in in major hubs of ascending and descending pain pathways [14,34]. In particular, we found profound alterations affecting connections form the contralateral amygdallar nuclei to cortical somatosensory (B1c, B2c, Lc) and limbic structures such as cingulate, temporal associative and insular cortex as well as the midbrain. Midbrain structures indicated altered connectivities mostly in motor-related areas, likely reflecting impaired motor function of the tumor-bearing limb. Seed-based analysis supported these findings and furthermore indicated altered connectivities between the anterior and posterior periaqueductal gray and cortical structures, which are directly linked through the ascending spinothalamic tract. Connectivities involving thalamic regions were found less affected. This could be attributed to the anesthesia regime comprising medetomidine, which is known to disrupt thalamo-cortical connectivity in fMRI experiments [15,19,38]. The regions mentioned above play important roles in nociceptive transmission and encoding of painful stimuli. As they were found to be unaffected upon prospective treatment with anti-NGF treatment, our study indicates the efficacy of such treatments for preventing FC alterations elicited by persistent pain. While hemispheric lateralization of the amygdala in emotional processing is well documented, its role in pain processing is still not fully understood. As pain comprises a strong emotional and affective component, the amygdala plays a critical role in modulating such components as well as in direct modulation of pain perception [8]. Neurons of the latero-capsular division of the central nucleus of the amygdala (CeLC) have been described to receive specific nociceptive inputs from different body regions in rats. The right amygdala receives input from deep tissue, both hindlimbs as well as the tail while left amygdala CeLC neurons respond mostly to nociceptive input in the contralateral hindlimb. Nevertheless, electrophysiological studies have found unique activation of the right amygdallar CeLC in response to induction of knee arthritis [28]. On the other hand, a transient increase in left amygdallar activity has been described in a neuropathic pain condition in vivo using electrophysiological recordings while at late stages of the model the right amygdala

was activated predominantly [17]. The discrepancy between those results and our study might arise from the nature of pain derived from bone metastasis which comprises several aspects such as nociceptive, neuropathic and inflammatory pain rather than being purely neuropathic. Additionally, processing of emotional components by amygdallar CeLC neurons has been described to occur predominantly on the right in male subjects, while in female subjects these processes occurred predominantly in the left amygdala [7]. More recent studies have also suggested that the right amygdala is predominantly involved in pro-nociceptive signaling, while the left amygdala processes anti-nociceptive signals [43]. Therefore, our findings might indicate a failing top-down suppression of nociceptive signaling at the last fMRI time point which has been described to be mediated by the infralimbic medial prefrontal cortex [41].

Cancer pain associated FC alterations could be successfully prevented through the prospective administration of anti-NGF antibody mAb911 with only few residual interactions. Seed-based analysis indicated no changes in connectivity strength as indicated by Z-scores as a function of time in Tumor+anti-NGF mice throughout the observation period. In fact, values were not different from those measured in Sham+Vehicle animals at all time points. This indicates on one hand the specificity of FC alterations for tumor-associated persistent pain, and on the other hand the effectiveness of mAb911 treatment in preventing these pathological changes. Additionally, control-seeds in the ipsilateral amygdallar nucleus did not show previously observed trends, endorsing the validity of obtained results and further underlining their specificity (Supplementary Figure 1, available at http://links.lww.com/PAIN/A659). Residual alterations, as observed in the ROI-based network analysis, were to be expected as behavioral readouts of pain (guarding) indicated weak but significant spontaneous pain behavior at 20d.p.i. in the Tumor+anti-NGF group. These effects are unlikely due to direct central effect of anti-NGF treatment as the comparison of Sham+Vehicle and Sham+anti-NGF mice displayed only minimal differences in FC patterns affecting Mb regions but not pain associated ascending and descending pathways. This is in line with reports stating that NGF target receptors are largely absent from the CNS except from basal forebrain cholinergic neurons, where low levels of TrkA expression was described [45]. The absence of central NGF receptors together with the low CNS penetration of IgG molecules suggest minimal non-specific effects on FC While anti-NGF treatment addresses important aspects of bone cancer pain such as neuropathic and inflammatory nociceptive processes, additional processes not related to NGF such as bone remodeling associated with tumor growth will trigger peripheral mechanical pain stimuli, which might lead to residual FC changes described.

Regarding behavioral readouts, we would like to mention that the difference between tumor-bearing and sham-operated animals regarding the number of flinches and the time spent guarding reported in this study appears low in absolute numbers. This can be attributed to the early time points of measurements (day 13 and 18 following tumor implantation), which have been chosen to investigated early changes in the brain functional architecture in response to

nociceptive input. In fact, the behavioral measures of the current study follow the trajectory of developing behavioral signs of pain that has been reported previously for this model [6], illustrating good reproducibility. Considering the effect size of behavioral symptoms between tumor-bearing and sham-operated animals, our results are in line with results obtained from a similar tibial bone cancer model, though using a different tumor cell line and mouse strain [29]. Differences in absolute values are not surprising as it has been reported that the extent of behavioral signs of pain depends on the mouse strain used [37] and almost certainly also on the osteolytic potential of the tumor cells. Therefore, comparisons of absolute numbers in behavioral readouts of nociception should be viewed with care.

Prospective treatment using mAb911 effectively prevented FC alterations observed in tumor-bearing untreated animals. It has been previously shown that anti-NGF treatment effectively reverses nerve sprouting, neuroma formation and nociceptive behavior upon preventive administration as well as under a therapeutic treatment regime in mice [29]. While preventive administration is certainly more effective as it blocks pathological nociceptor alterations before they occur, it typically does not represent the clinical situation. Nevertheless would preventive administration most likely be desirable also in a clinical setting in order to effectively prevent such pathological events, thereby counteracting development of a pain state from bone metastasis in affected patients. Thereby, efficacy of anti-NGF antibody mAb911 to reverse FC alterations after their development could be assessed in future studies.

In summary we could show that anti-NGF treatment using mAb911 not only effectively prevents peripheral nociceptive processes but also effects on FC of the brain, as indicated by rs-fMRI. This supports previously published promising effects of anti-NGF antibody mAb911 to effectively prevent and treat cancer-induced skeletal pain.

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Conflict of Interest Statement

None of the material contained in this manuscript has been published or presented previously, except in abstract form at international conferences. This paper has not been submitted for publication elsewhere, and it has been reviewed and approved by all the authors. There are no financial or other relations that could lead to a conflict of interest.

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Figure Captions

Figure 1 | Experimental design. Resting-state fMRI experiments were carried out -2, 10 and 20d.p.i.. At 0d.p.i., mice of the tumor groups were injected with EO771 breast cancer cells (T) control mice with PBS (C). Guarding and flinching (G/F) behavior was assessed on the same days as application of treatment was performed, treatment-groups receiving anti-NGF mAb911 (10mg/kg, i.p.) while vehicle groups received physiological saline instead.

Figure 2 | Behavioral readouts of spontaneous pain behavior. Guarding and flinching was assessed during a period of 2min. Statistical significance was tested post-hoc per timepoint compared to Sham+Vehicle using t-tests accounting for multiple comparisons (Holm-Sidak method). Brackets indicate significant treatment-effects, comparing Tumor+anti-NGF to Tumor+Vehicle (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$)

Figure 3 | ROI-based network analysis of longitudinal rs-fMRI readouts. Pair-wise correlations between different ROIs were calculated for all sessions. Alterations in persistent pain and effects of treatment were tested for statistical significance using a linear mixed model analysis, testing for significant longitudinal group and session interaction. Respective p-values are plotted in a heatmap. The upper triangle indicates significant differences between Tumor+Vehicle and Sham+Vehicle animals. The lower triangle represents the comparison between Tumor+anti-NGF and Sham+Vehicle animals, i.e. the effect of the treatment on FC, indicating only little differences between the two groups.

Figure 4 | Seed based correlations were analyzed in regions of interest, plotting Z-scores as group averages. Projections of the left amygdallar seed (L Amg) to the thalamus (Th), dorsal hippocampus (dHp) and the barrelfield 1 cortex (B1c) indicate FC changes in Tumor+Vehicle animals, while other groups were not altered. Furthermore, projections of the right superior colliculus seed (R SCm) to the amygdalar RNS, anterior periaqueductal gray (aPAG) seed to the barrelfield 2 cortex (B2c) RNS and posterior peraqueductal gray (pPAG) seed to the limb cortex RSN were found to be altered in Tumor+Vehicle animals only. Statistical significance was assessed in a linear mixed model testing for group and session interaction as well as for a group effect in session 3 in order to assess a significant treatment effect (* $p<0.05$, ** $p<0.01$).

Figure 5 | Visualization of FCs derived from seed-based analysis that exhibited significant difference when comparing sham-operated mice with animals displaying persistent pain from bone cancer as revealed by behavioral tests. All these FC changes could be successfully prevented by treatment with the monoclonal antibody mAb911. Individual seeds used to extract z-scores are color coded as are the FCs originating from the respective seed. Effect-size of functional alterations are indicated as differences in Z-scores (ΔZ) comparing Tumor+Vehicle vs Sham+Vehicle animals. No directionality can be inferred from the correlation analysis. P-values for the individual FCs are given in text.

Figure 6 | FC differences in affected resting-state networks as deduced by seed-based analysis at 20d.p.i.. Uncorrected t-statistical maps visualizing increasing seed FC within RSN in Tumor+Vehicle compared to Sham+Vehicle animals are indicated in the left column, while Tumor+anti-NGF compared to Sham+Vehicle animals are depicted in the right column. Efficacy of mAb911 treatment to prevent FC alterations is indicated through a reduction of altered FC in the Tumor+anti-NGF compared to the Tumor+Vehicle group. Significance of differences between groups are indicated in a heatmap representing t-statistics on a voxel-wise basis.

Experimental Group	Surgical Procedure	Treatment	Group-Size
Sham+anti-NGF	10µl PBS, intramedullary	mAb911, 10mg/kg, i.p.	N=7
Sham+Vehicle	10µl PBS, intramedullary	50µl Saline	N=7
Tumor+anti-NGF	10^5 EO771, intramedullary	mAb911, 10mg/kg, i.p.	N=8
Tumor+Vehicle	10^5 EO771, intramedullary	50µl Saline	N=8

Table 1 | Experimental groups as referred in the text. All surgical procedures were performed on the right tibia. mAb911 was diluted in 50µl physiological saline before injection.